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13. ABSTRACT *(Maximum 200 Words)*

This is the final report documenting the achievements of DAMD17-96-1-6097 "Role of seprase in breast cancer invasion". The goals were to produce full length seprase cDNAs and use these to produce cells that overexpress seprase. These seprase expressing cells were to be evaluated for invasive behavior relative to those that do not express seprase. The major goals of the project have been accomplished with the exception of engineering seprase-negative breast cancer cells to express active seprase. However, it was possible to evaluate the role of seprase in invasion by suppressing seprase expression in breast cancer cells that express seprase to a high level. Seprase suppressed transfectants of MDA-MB-436 and MDA-MB 435 breast cancer cells that express seprase were produced and compared to parental cells that express seprase in matrix proteolysis and invasion assays. These experiments suggested that seprase does not have a major role in promoting matrix degradation, invasion and metastasis. However, the experiments revealed that seprase has an important role in promoting breast cancer cell proliferation. These novel findings, described in this report, are the first to implicate seprase in breast cancer cell growth. The research has resulted in 3 papers, 1 manuscript, and 3 abstracts.

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Introduction

This is the final report summarizing the accomplishments of the four year project entitled "Role of seprase in breast cancer invasion". Seprase is a serine integral membrane protease (1) first identified in human melanoma cells (2) and chicken embryo fibroblasts transformed by Rous sarcoma virus (3). In 1997, a cDNA encoding human melanoma seprase was cloned and sequenced by others (1). The sequence reveals that seprase is a member of a family serine integral membrane proteases that includes fibroblast activation protein-α (FAP-α) (4), and dipeptidyl peptidase IV (DPPIV) (5,6). Seprase is known to degrade gelatin (1-3) and is thought to facilitate erosion of the extracellular matrix thereby promoting invasion of malignant cells. Supporting this concept is the association of seprase overexpression with the invasive phenotype and its concentration on the invadopodial membranes of human melanoma cells (2,7). Moreover, accumulation of seprase on the surface of invadopodial membranes is stimulated by ligation of $\alpha_6\beta_1$ integrin on the melanoma cell surface (8). The localization of seprase to invadopodia supports a role for seprase in degrading extracellular matrix and facilitating tumor cell invasion because invadopodia are specialized protrusions of the plasma membranes of invasive cells that contact and degrade extracellular matrix (9,10). Invadopodia can cause proteolysis of intact fibroblast extracellular matrices, type I collagen, type IV collagen, laminin, and fibronectin (3). Several malignant cell types are known to use invadopodia to degrade extracellular matrix including, human melanoma cells (2,7,10), Rous sarcoma virus-transformed chicken embryo fibroblasts (3,9,11) and human breast cancer cells (12-14). The fact that malignant human breast cells degrade extracellular matrix with invadopodia suggested that seprase might have a role in promoting the invasive spread of human breast cancer.

Seprase is overexpressed by invasive human breast cancer (15). This conclusion is now based on four lines of evidence published or being prepared for publication as the direct result of the funding of this project. Specifically, a seprase-specific polyclonal antibody produced using affinity-purified chicken embryo seprase intensely labels malignant breast cells but not normal breast epithelia or stromal cells (15). In addition, seprase activity was detected by zymography in extracts of human breast cancer tumors and the seprase activity was five times greater than that of chicken embryo seprase as determined using a ^{3}H-gelatin substrate (16). Seprase activity was detected by zymography in extracts of human breast cancer cell lines but not in extracts of a normal human breast cell line. Moreover, we reported in Annual report September 1999, direct evidence for the over expression of seprase by human breast cancer cells. Thus we believed that seprase has an important role in breast cancer cell invasion. The project sought to investigate the role of seprase in breast cancer cell invasion by these specific aims:

1) Identify and characterize a full-length cDNA for human breast cancer seprase.

2) Investigate the contribution of seprase to the metastatic potential of breast cells.

Achievements made towards completing these objectives is described in the body of this final report and previous annual reports in relation to the statement of work submitted with the original proposal. The work completed over the past year has dramatically changed our thinking as to the role of seprase in breast cancer. We now believe seprase promotes growth of breast cancer cells.

Body

Personnel have remained the same since the 1999 report

Johnna D. Goodman is pursuing her Ph.D. on this project. Johnna led the effort to characterize the phenotype of transfectants of MDA-MB-436 and MDA-MB-435 breast cancer cells that are down-regulated in seprase activity. Johnna's student stipend is not supported by the DoD monies.

Personnel supported by the DoD grant

Tricia Rozypal is a Research Technologist II who devotes 100 % of her effort towards this project. Ms. Rozypal completed site-directed mutagensis of our original seprase cDNA clones to produce wild-type seprase cDNA. She put the wild type construct into the pcDNA 3.1- mammalian expression vector and attempted to transfect MCF-7 cells. This has proven problematic and we will try a retroviral vector soon. However, she has also transfected MDA-MB-231 human breast cancer cells with wild type seprase cDNA. Ms. Rozypal's entire salary and benefits are provided by the DoD monies.

Thomas Kelly, Ph.D., is the PI on the project and continues to devote 60 % of his time towards data analysis and interpretation, deciding experimental strategies, writing and publishing the findings, and performing experiments pertinent to this project. The DoD monies provide 60 % of Dr. Kelly's salary and fringe benefits.

Progress in year 4 and for the whole project period.

SOW Task 1. Months 1-3: Produce cDNA expression library with mRNA purified from human breast tumors. Complete

As discussed in earlier reports, we have already produced high quality chicken embryo cDNA expression libraries. We have focused our efforts on RT-PCR cloning of the human breast cancer seprase cDNA from the breast cancer cell line MDA-MB-436 as described below.

SOW Task 2. Months 1-36: Produce additional seprase-specific probes for screening the cDNA expression library. Complete

I) Oligonucleotide probes based on seprase, fibroblast activating protein-α (FAP-α) and dipeptidyl peptidase IV (DPPIV) sequences. Completed as described in 1998 and 1999 annual reports.

II) Antibodies to seprase, As reported in the 1999 annual report, sequence analysis of seprase-specific clones reveals that this protein is identical to the previously cloned fibroblast activation protein-alpha (FAP-α). Thus we have used this publicly available monoclonal antibody to FAP-α (F19) to investigate seprase expression in human breast cancer cell lines.

SOW Task 3. Months 3-18: Identify clones with full-length seprase cDNA inserts. Completed as described in the 1999 annual report.

SOW Task 4 Months 4-24: Confirm full-length seprase cDNA clones. Complete.

The seprase 2.4 kb and 1.1 kb cDNAs have been cloned and sequenced. Thus this task is complete as documented in the 1999 report. However, as we reported in 1999, none of the clones was a perfect wild type. We chose one cDNA clone (clone 49) with a single point mutation as the starting point to engineer a wild type seprase cDNA. A site directed mutagenesis procedure was performed to produce a cDNA encoding "wild-type" human breast cancer seprase as described in 1999. A 2.4 kb putative wild type seprase cDNA was produced by the site-directed mutagenesis procedure. This cDNA was successfully cloned into the pCR2.1 cloning vector and sequence analysis has confirmed the successful production of wild type seprase cDNA (not shown). This wild type seprase cDNA has been cloned into the pcDNA 3.1 mammalian expression vector and used to transfect MCF-7 cells as described below. Thus, we have produced wild type seprase cDNA, three different full length but mutated seprase RT-PCR mutants, and 5' truncated form that may represent as documented in 1999.

SOW Task 5 Months 18-36: Produce stable transfectants of malignant and normal breast cells that overexpress assembled, active seprase to the cell surface. Task 5 months 18-36 Complete: Failed by original plan, but succeeded with an alternate plan as described below

The purpose of this task was to produce genetically related cell populations that differed from each other with respect to seprase expression. The consequences of seprase expression on malignant behavior could be determined by comparing the two closely related cell populations. We have succeeded in the overall goal of this task, but by a method different than that originally proposed. Specifically, we had planned to engineer MCF-7 cells to express high levels of fully active seprase. However, as described below, we have failed to produce stable lines of seprase expressing MCF-7 cells. Thus, we decided to knock out seprase expression in breast cancer cells that express active seprase to the cell as described below.

Transfections of cells MCF-7 human breast cancer cells with seprase in the sense orientation. Stable transfection of MCF-7 human breast cancer cells has not succeeded for reasons that are not clear. In the 1999 report we showed that after transfection with any one of three different seprase cDNAs, MCF-7 cells grew in the selection medium but did not have detectable levels of cell surface seprase (1999 report, figure 12). We speculated that the lack of cell surface expression was due to mutations in our seprase cDNAs. Since the last report, but prior to producing the wild type seprase cDNA, we expressed active seprase in MCF-7 cells using cDNA clone 48 that has a fifteen amino acid deletion (1999 report). Seprase cDNAs in the pcDNA 3.1 mammalian expression vector (Invitrogen) were transfected into MCF-7 cells using lipofectamine and the cells were selected by prolonged exposure to G418. Seprase activity was readily detected by gelatin zymography in MCF-7 cells transfected with seprase (Appendix, Fig. 1, 48). Unfortunately, this seprase activity was soon lost over a brief time in culture and we were unable to clone the transfected cells expressing seprase. It was not clear why the seprase-expressing transfectant was lost. At the time, that we lost the seprase expression, the original grant period performance was nearly over. We requested were granted a 6 month a no cost extension of the project to produce MCF-7 cells that express seprase using the wild type seprase cDNA.

We have tried and failed several times to produce stable transfectants of MCF-7 cells that overexpress seprase with wild type seprase cDNA in the pcDNA3.1 mammalian expression vector. We now believe that transfection with a mammalian expression plasmid was not the best choice. Others have reported anecdotal evidence that MCF-7 cells can be difficult to produce stable transfectants that continue to express the introduced cDNA using plasmid expression vectors (personal communication Dr. Therese Guise, San Antonio, TX). Thus, despite receiving a six month no cost extension, we have not succeeded in producing stable transfectants of MCF-7 cells that overexpress seprase. In future, we plan to produce the seprase expressing MCF-7 cells by using retroviral vectors. These vectors will insert multiple copies of the seprase cDNA at many different sites in the genome. However, we have also transfected MDA-MB-231 cells with the wild type seprase cDNA in the sense orientation. Unlike the MCF-7 transfectants, these cells are growing well in the selection medium and we will soon be able to test their expression of wild type seprase.

Down-regulation of cell-surface seprase by transfections of MDA-MB-436 and MDA-MB-435 cells with seprase cDNAs in the antisense orientation.

Because, the MCF-7 transfection work was not progressing on schedule we proceeded to investigate the role seprase of seprase in breast cancer using seprase suppressed breast cancer cells produced by transfection of cells with antisense seprase cDNA. Seprase is expressed to high levels by MDA-MB-436 and MDA-MB-435 cells (see 1999 report, Figs. 10 & 11). Seprase expressing MDA-MB-436 were transfected with seprase cDNA in the antisense orientation. As reported in the 1999 report, we produced seprase-suppressed transfectants of the MDA-MB-436 cells (1999 report, Fig,

14; see also this report appendix Fig. 2). Since the last report, we also produced seprase suppressed transfectants of the MDA-MB-435 human breast cancer cell line (Appendix, Figs. 2 & 3). Sequence analysis revealed several mutations in seprase cDNA clone 56 that were apparently introduced arbitrarily by the RT-PCR cloning procedure (see 1999 report). This cDNA clone proved useful for antisense suppression of seprase. Seprase cDNA clone 56 was inserted into pcDNA3 for expression in the antisense orientation. Antisense transfectants of MDA-MB-436 human breast cancer cells were obtained that exhibited a marked down-regulation of seprase expression (Appendix, Fig. 2A & B). Seprase (170 kDa) was readily detected by immunoblotting with F19 mAb in extracts of parental MDA-MB-436 cells, stable vector-only transfectants and antisense cDNA clone 48 stable transfectants (Appendix, Fig. 2A, Parental, Vector-E8, & 48AS). In contrast, the clone 56 antisense stable transfectants and a clonal transfectant of these cells, termed 56H5, did not have seprase in extracts as judged by Western blotting with the F19 mAb (Appendix, Fig. 2A, 56AS, 56H5AS). Seprase activity was also lowered considerably as judged by zymography of identical extracts of antisense transfectants of MDA-MB-436; 56 and 56H5 (Appendix, Fig. 2B, 56 & 56H5). But as expected, seprase gelatinase activity was detected in parental MDA-MB-436 cells, stable vector-only transfectants, and the antisense 48 stable transfectant (Appendix, Fig. 2B, Parental, Vector-E8, & 48AS).

Similar results were also obtained for stable transfectants MDA-MB-435 breast cancer cells using clone 56 expressed in the antisense oritentation (Appendix, Fig 2C & D). Immunoblot analysis with mAb F19 revealed high levels of seprase (170 kDa) in extracts of the parental MDA-MB-435 cells (Appendix, Fig. 2C, Parental). Reduced seprase was detected in extracts of cells transfected with clone 56 in antisense orientation (Appendix, Fig. 2C, 435AS56). High levels of seprase activity were found by zymography in MDA-MB-435 cells (Appendix, Fig. 2D, Parental) but lower activity was found in the antisense transfectants (Appendix, Fig. 2D, 435AS56). It should be noted that MDA-MB-436 and MDA-MB-435 antisense transfectants are not clonal transfectants and as such represent a mixed population of cells. Only the 56H5 transfectant of the 436 cells is a clonal transfectant.

FACS analysis of cell-surface seprase expression with mAb F19 reveals that the MDA-MB-435 parental cells express a high level of seprase on their surfaces (Appendix, Fig. 3A). This is consistent with the biochemical results (Appendix, Fig. 2) and with the results found for the MDA-MB-436 cells (1999 annual report). The MDA-MB-435 antisense cDNA clone 56 transfectants reveal a reduction in cell-surface seprase levels (Appendix, Fig. 3B). Thus, antisense expression of clone 56 reduced seprase expression by MDA-MB-435 cells.

In summary, we have succeeded in producing seprase expressing and seprase suppressed cells but in a manner different from that originally proposed. Instead of introducing active seprase into cells that do not express it, we removed active seprase from those that express it. Two different pairs of genetically related stable breast cancer cell lines have been produced. Each pair consists of a seprase expressing wild

type and control transfectants as well as seprase suppressed transfectants. We demonstrated the cell surface expression and the proteolytic activity of the seprase produced by the parental and control transfected cells. We also demonstrated that seprase expression has been greatly decreased in the antisense transfectants. The seprase expressing MDA-MB-436 and MDA-MB-435 cells and their seprase suppressed transfectants, were used to investigate the role of seprase in breast cancer matrix proteolysis, invasion, tumorigenicity, growth and metastasis (SOW tasks 6 & 7). Experiments were performed as originally proposed except that seprase expressing MDA-MB-436 and/or MDA-MB-435 cells and their seprase suppressed counterparts were substituted for MCF-7 and MCF-7 transfected cells expressing seprase.

SOW Task 6. Months 24-48: Determine effects of seprase overexpression on cell-mediated matrix proteolysis. Complete

This task has been completed. We were unable to demonstrate a role for seprase in degradation of the matrix by either MDA-MB-436 cells or MDA-MB-435 cells. These experiments were performed by growing the cells on fluorescently labeled fibronectin substrates covalently linked to a glutaraldehyde cross-linked gelatin film as we described in a published report from this work (14). After growing on the films for 24 to 72 hours, the cells were fixed and the fluorescent matrix films inspected by fluorescence microscopy (14). Degradation of the extracellular matrix was visualized as fluorescence negative spots underneath cells that were actively degrading the matrix (14). The results were disappointing. In the published report, the aggressively invasive MDA-MB-231 cells were used to degrade the matrix, which they do quite efficiently (14). Unfortunately, these cells do not express seprase so we could not remove seprase from them to observe potential effects of seprase on matrix degradation.

The MDA-MB-436 and MDA-MB-435 cells that express seprase do not perform well in this assay. Specifically, in over 80 % of experiments (greater than 10 attempted for each cell line) no evidence of matrix degradation could be detected in either the seprase expressing parental cells or the seprase suppressed transfectants. Moreover, in the minority of cases where evidence of matrix degradation was observed, it was a rare occurrence. Usually, less than 1 in 10 microscopic fields observed with the 60X objective. In fact, it was the inability of the seprase suppressed MDA-MB-436 cells and there seprase expressing counter parts to degrade extracellular matrix and their poor invasion into collagen gels (described below) that prompted us to produce the seprase suppressed MDA-MB-435 cells. Our hope was that these cells would perform better in the matrix degradation and collagen invasion assays.

Our results have led us to conclude that seprase is not critical for extracellular matrix degradation as we initially believed. Several lines of evidence point to this conclusion. First, seprase is not expressed by the MDA-MB-231 cells that efficiently degrade the extracellular matrix. Moreover, though seprase is a serine protease and

the matrix degradation by MDA-MB-231 cells is inhibited by matrix metalloproteinase inhibitors (14). This suggests that seprase is not necessary for matrix degradation to occur and that matrix metalloproteinases may play a greater role in degrading the extracellular matrix. Secondly, neither of two breast cancer cell lines that express seprase to high levels shows good evidence of matrix degradation. Finally, we have evidence to suggest that the main role of seprase is to promote cell growth (described below).

SOW Task 7. Months 30-48: Determine effects of seprase overexpression on breast cell invasion of extracellular matrix. Complete

We have completed the in vitro and in vivo invasion experiments that were proposed. For these experiments we using the MDA-MB-436 and MDA-MB- 435 cells and their seprase suppressed counter parts for in vitro invasion assays, as well as the MDA-MB-436 panel of cells for an in vivo experiment to evaluate invasion and growth. These experiments were inconclusive primarily because neither the seprase expressing cells or their seprase suppressed counterparts invaded consistently in the in vitro invasion assays and only one group of animals developed tumors in the in vivo experiment. These inconclusive experiments are briefly reported below. In the course of these experiments we began to investigate other potential roles for seprase in the pathobiology of breast cancer. Three different but possibly related findings that suggest a role for seprase in promoting breast cancer cell growth are presented in this section. This growth promoting role for seprase was unexpected and is a novel and potentially very important finding of the study.

Investigation of invasiveness of seprase expressing and seprase suppressed human breast cancer cells.

Type I collagen invasion assays These experiments were performed as described earlier (14). Parental MDA-MB-436 cells and the vector-only clonal transfectants of MDA-MB-436 (vector-only E8) having high levels of seprase, were evaluated for invasiveness together with the 56436 stable polyclonal transfectants and the 56E10 and 56H5 antisense clonal transfectants of these cells with suppressed seprase. MDA-MB-435 parental cells as well as the stable polyclonal transfectant of these cells were also tested for invasion.

Cells (5×10^6) were seeded in duplicate onto type I collagen gels (0.5 mg/ml) that were polymerized in serum-containing cell growth media. After 48 h of growth at 37^0 C, invasion was evaluated by removing the non-invading cells on top of the collagen gel with trypsin-EDTA and mild collagenase treatment. When removal of the non-invading cells was complete as judged by phase contrast microscopy, the invading cells were harvested after dissolving the gels with collagenase.

In 85 % of invasion experiments with MDA-MB-436 (greater than 10 total experiments) none of the cells showed significant invasion. The MDA-MB-435 cells never showed significant invasion in this assay over 10 experiments. Significant invasion was arbitrarily defined as that where 10 % of the cells or more invaded. The background for the assay varies considerably and is 1-3 % invasion. Two examples of results from MDA-MB-436 invasion experiments are presented where significant invasion was observed.

Experiment performed 12/23/99 invasion was observed in parental MDA-MB-436 cells (11.95 % invading cells, range 8.4-15.5 %) and the E8 vector-only clonal transfectant (10.1 % invading cells, range 9.4-10.7 %). The 56436 seprase suppressed cell line revealed invasion comparable to the seprase expressing cells (9.8 % invading cells, range 8.3 - 11 %) as did the seprase suppressed clone 56E10 (12.3 % invading cells, range 8.4-16.2 %). The 56H5 clonal transfectant with seprase suppressed cells showed reduced invasion (4.8 % invading cells, range 3.4-6.2 %) relative to the seprase expressing cells but this difference did not achieve statistical significance.

An experiment performed on 1/8/00 revealed unusually high invasion in all cases, but again the invasion in all but one case were comparable to each other. In this experiment wild type seprase expressing cells invaded the least successfully (8.6 % invading cells; range 5.7-11.4 %) while all others E8 vector only seprase expressing cells (41 % invading cells; only one well tested) and seprase suppressed 56H5 cells invaded to high levels (17.7 % invading cells, range 17.6-17.7). Again, the results were inconclusive.

Difficulties with getting these cells to invade in approximately 85 % of these assays, coupled with failure to achieve statistically significant differences between seprase expressing and suppressed cells when invasion was observed, has left us unable to demonstrate that seprase has a role in breast cancer cell invasion.

Animal experiment: Using the antisense seprase transfectants, we have begun an animal experiment to determine the role of seprase in breast cancer cell invasion. Four groups of seven Balb c nu/nu mice have been purchased and given 60 day estrogen release pellets as described in the original proposal. Each mouse has been injected ID into the mammary fat pads at 4 different sites with 2 x 10^6 cells per site. The groups are mice injected with: 1) MDA-MB-436 wild type cells; 2) partial seprase down-regulated MDA-MB-436 56E10 antisense transfectants; 3) MDA-MB-436 3.1E8 control transfectants and 4) total seprase down-regulated MDA-MB-436 56H5 antisense transfectants. After 11 days the tumor take appeared very good with groups 1,2 & 3 having X tumors per 28 injection sites. After 12 days the animals in group 4 have only a 50 % tumor take, consistent with the notion that these cells do not grow as well as wild type and control-transfected MDA-MB-436 cells. With time the numbers were much worse. Only 4 animals in group 1 survived and just one of these developed a single very large (3.77 g), well encapsulated tumor with no obvious invasion or metastasis. The other three mice injected with wild type MDA-MB-436 cells had no visible tumors after 154 days. Group 2 animals had excellent tumor take (23 tumors/28

sites, 7 mice). Only the mice receiving the 56E10 cells formed significant numbers of tumors that grew over the period of the experiment. These tumors were harvested at day 68 and were relative large (0.06g - 1g). Tumors of 56E10 cells were evaluated by a pathologist (Dr. Irene Garner, University of Arkansas for Medical Sciences) and found to be encapsulated, with necrotic centers. There was no indication of tumor invasion into the surrounding tissues. Organs were dissected but there was no evidence of metastasis. Group 3 animals received the vector-only control E8 cells and three mice did not develop tumors even out to 153 days. Two mice developed tumors that grew well. These mice were harvested on day 68 with a tumor take of 6 tumors/8 sites of these two but an overall tumor take of 6 tumors/20 sites. The tumors were small (0.007 g - 0.038g), encapsulated with no obvious invasion. Group 4 with tumors of 56H5 seprase suppressed cells had a poor tumor take. Of the four mice that survived only one large tumor was found after 155 days. Again this tumor was well encapsulated and showed no signs of invasion. Thus, these seprase suppressed cells had a very poor tumor take (1 tumor/16 sites). Overall it is impossible to draw any conclusions about invasion because no tumor in any of the four groups exhibited local invasion or metastasis. The 56E10 with partial seprase suppression grew very well while all others did not grow well. It is also impossible to draw any firm conclusions about growth.

Overall the matrix proteolysis results, the invasion assays, and the animal studies are consistent with the view that seprase does not have a major role in invasion and metastasis. We have not ruled out a role for seprase in invasion. However, it appears that if seprase has a role in breast cancer cell invasion then it is likely to be more of a subtle and facilitating role rather than a function that is absolutely necessary for invasion to proceed. This suggested that perhaps invasion itself was not the main role of seprase. Thus we began looking for other ways in which the seprase suppressed cells might differ from their seprase-expressing counterparts.

Novel role for seprase in promoting growth of breast cancer cells

Seprase-suppressed breast cancer cells exhibit an more aggregated morphology on type I collagen than their seprase-expressing counterparts. While attempting to measure invasion using type I collagen gels we noticed differences in morphology between seprase expressing and seprase suppressed breast cancer cells. On type I collagen, wild type MDA-MB-435 seprase-expressing breast cancer cells tend to grow as individual cells or in loose aggregates or clumps of cells where the individual cells comprising the clumps are clearly distinct from each other (Appendix, Fig. 4A). This is as compared to seprase-suppressed MDA-MB-435 cells that grow in islands and balls of closely associated and fused cells (Appendix, Fig. 4B, arrows). Though the precise meaning of this morphological change has not been determined, the close cell-cell association of the seprase-suppressed cells may represent a switch towards a more normal phenotype because normal breast epithelial cells form tight adhesions with each other. The morphological distinction between seprase-expressing and seprase-

suppressed cells likely results from altered signaling and is consistent with seprase expression maintaining the malignant phenotype of the MDA-MB-435 cells.

Seprase promotes cell growth under serum free conditions MDA-MB-435 and MDA-MB-436 parental cells, and seprase antisense transfectants were grown in cell growth media with 5 % fetal bovine serum or in cell growth media lacking fetal bovine serum. Viable cell number was determined in triplicate using the "cell proliferation assay" (Promega) and measuring absorbance at 492 nm at 0h, 24h, and 48h time points. In normal growth media and 5 % fetal bovine serum, growth curves were similar for both MDA-MB-435 cells expressing seprase (Appendix, Fig. 5A, left panel, seprase high) and cells with suppressed seprase expression (Appendix, Fig. 5, left panel, seprase low) with cell numbers increasing over the two days of the experiment. In serum free medium, the numbers of parental MDA-MB-435 cells expressing high levels of seprase increased steadily over the two days growth without serum (Appendix, Fig. 5A, right panel, seprase high). In contrast, MDA-MB-435 cells with suppressed seprase levels showed no statistically significant change in cell number over 48 h following removal of serum (Appendix, Fig. 5A, right panel, seprase low). Similar results were obtained when comparing MDA-MB-436 cells expressing high and low levels of seprase (Appendix, Fig. 5B). Seprase expressing and seprase suppressed cells grew in serum-containing medium (Appendix, Fig. 5B, left panel, 436 seprase+; 56H5 436 seprase-). However, in serum free medium, seprase expressing cells continued to grow, albeit slowly, (Appendix, Fig. 5B right panel, 436 seprase+) while seprase suppressed cells did not proliferate (Appendix, Fig. 5B right panel, 56 436 seprase-).

Serum starvation blocks seprase-suppressed cells in G_2 of the cell cycle. Cell cycle analysis of propidium idodide stained cells was performed to begin to evaluate possible causes for the lack of growth observed for serum-starved breast cancer cells. Cells were plated in serum containing medium and allowed to attach to the culture flask for one hour at 37^{0}C. The medium was removed, the cells washed three times with sterile PBS, and then grown in serum-free medium. Cells were released from the flask with trypsin and fixed in ethanol after growing in serum free medium for 24 hand 48 h. The cells were stained with propidium iodide and evaluated for DNA content by fluorescence activated cell sorting. Seprase expressing parental MDA-MB-436 cells continued to grow normally under serum-free conditions. 56 % of cells were in G_1 and 16 % were in G_2 after 24 h with 16 % cells in S (Appendix Fig. 6A). Similar results were observed for seprase expressing MDA-MB-436 cells grown under serum free conditions for 48 h. After 48 h, 64 % seprase expressing cells were in G_1 and 20 % in G_2 with 16 % cells in S indicative of normally proliferating cells (Appendix Fig. 6B). Seprase cells reveal a very different phenotype. These cells accumulate in G_2 over time while growing without serum. The 56436 line, a seprase suppressed, multiclonal line reveals 91 % cells in G_2 after 24 h (Appendix, Fig. 6C) and 48 h (Appendix, Fig. 6D) growth without serum. Similarly, the seprase suppressed 56H5 clonal transfectant of these

cells reveals 84 % cells in G_2 after 24 h (Appendix, Fig. 6E) and 48 h (Appendix, Fig. 6F) growth without serum. Seprase suppressed cells growing without serum have apparently completed S phase with less than 0.6 % or fewer cells in S phase 24 and 48 h after serum removal. These results indicate that the reason seprase suppressed cells do not grow under serum free conditions is that they become blocked in G_2. Moreover, it provides evidence that seprase promotes growth by enabling cells to overcome a G_2 block.

Seprase promotes survival of breast cancer cells treated with doxorubicin. Doxorubicin is a front line chemotherapeutic for breast cancer. Thus we investigated whether seprase expression altered the cellular response to doxorubicin. Seprase expressing MDA-MB-436 human breast cancer cells (Appendix, Fig. 7, left four clusters) and two seprase suppressed cell lines (Appendix, Fig. 7 middle & right clusters). The seprase expressing, wild type cells grew well under all concentrations of doxorubicin The two different seprase suppressed cells show increased sensitivity to doxorubicin. Both seprase suppressed cells were sensitive to 3 μM doxorubicin and failed to proliferate over 72 h (Appendix, Fig. 7, 56 seprase- 3 μM; 56H5 seprase- 3 μM). However these cells grew in the presence of 0.3 μM and 0.03 μM doxorubicin. Apparently suppression of seprase renders breast cancer cells more sensitive to doxorubicin. This finding could have important clinical implications for predicting response to chemotherapeutic agents such as doxorubicin.

Key Research Accomplishments this year:

- Confirmed cloning of wild type seprase cDNA
- Transfection of MDA-MB-231 human breast cancer cells with wild type seprase.
- Down-regulation of seprase by transfection of human MDA-MB-435 breast cancer cells with seprase cDNA expressed in the antisense orientation.
- Completion of in vivo and in vitro invasion studies
- Identification of an unusual aggregated morphology of seprase suppressed cells growing on type I collagen.
- Demonstration that seprase suppressed cells fail to grow in serum-free medium but that their seprase expressing counterparts grow well.
- Demonstration that seprase suppressed cells become blocked in G2 when serum-starved while seprase expressing cells reveal normal cell cycle kinetics.
- Seprase suppressed cells are apparently more sensitive to doxorubicin than their seprase expressing counterparts.

Additional key research accomplishments over the entire project:

- A completed study showing that seprase is over expressed in invasive tumor cells of human breast cancers (Kelly et al, 1998, Mod. Pathol., 11:855-863.
- A completed study (Kelly et al, 1998, Clin. & Exp. Metastasis 16:501-512)

showed that human breast cancer cells require functional invadopodia to degrade extracellular matrix and invade.

- Kelly et al, 1998, Clin. & Exp. Metastasis 16:501-512 also established the cell-mediated matrix proteolysis assay and *in vitro* invasion assay as well as providing baseline determinations of these parameters in the human breast cells.
- Production of a full length human breast cancer seprase cDNA by RT-PCR.
- A completed study establishing an assay for the gelatinase activity of seprase (Kelly, 1999, Clin.& Exp. Metastasis 17:57-62.
- Characterization of a partial cDNA for chicken embryo seprase
- Production of a fusion protein of chicken embryo seprase-calmodulin binding protein.
- Molecular cloning of a full-length 2.4 kb seprase cDNA from human breast cancer cells.
- Molecular cloning of a 1.1 kb seprase cDNA from human breast cancer cells
- Complete nucleic acid sequence analysis of the seprase cDNAs.
- Direct demonstration of seprase over-expression by Northern blot analysis of human breast cancer cell lines.
- Demonstration of functional seprase protein in naturally occurs breast cancer cell lines by immunoblot and zymographic analyses.
- Successful down-regulation of seprase by transfection of human breast cancer cells with seprase cDNA expressed in the antisense orientation.
- Initiation of animal studies of breast cancer cell growth and invasion using wild type, control transfectants, and transfectants of human breast cancer cells with decreased seprase expression.

Reportable Outcomes:
Since last report:

Results presented at the Era of Hope meeting 2000, Atlanta, GA.

Seprase facilitates invasion and survival of human breast cancer cells Johnna D. Goodman, Tricia L. Rozypal and Thomas Kelly Department of Pathology, Arkansas Cancer Research Center, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7199

Abstract

Seprase is a cell surface, serine protease that degrades extracellular matrix and may function in tumor cell invasion. Zymography, immunoblot, and northern blot analyses reveal that seprase is expressed to high levels by two invasive human breast cancer cell lines (MDA-MB-435 (435) and MDA-MB-436 (436)). However, seprase is

not expressed by non-invasive MCF-7 human breast cancer cells or by the invasive MDA-MB-231 human breast cancer cells. Seprase was suppressed in the 436 and 435 cells by antisense seprase cDNA expression. Seprase antisense transfectants of 436 cells were cloned and complete suppression of seprase was determined by fluorescence-activated-cell-sorting, immunoblotting, and zymography. Seprase-suppressed 436 cells were considerably less invasive in type I collagen gels than parental cells or control transfected cells with high levels of seprase. These results provide direct evidence that overexpression of seprase by human breast cancer cells facilitates their invasion into extracellular matrix *in vitro*. Suprisingly, the seprase suppressed cells exhibited increased serum-dependence. Seprase-expressing and seprase-suppressed cells proliferate in serum-containing media. However, seprase-suppressed cells stop proliferating over 48 h of serum starvation, while seprase-expressing cells continue to proliferate. These findings suggest seprase promotes growth of breast cancer cells.

Abstract of manuscript in preparation:

Seprase Promotes Growth Of Human Breast Cancer Cells Johnna D. Goodman, Tricia L. Rozypal, and Thomas Kelly, Department of Pathology, Arkansas Cancer Research Center, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7199

Summary

Seprase is a cell surface, serine protease that is expressed to high levels by invasive cancer cells of infiltrating ductal carcinomas of the breast but its function is unknown. MDA-MB-435 and MDA-MB-436 human breast cancer cells express seprase to high levels as do the carcinoma cells in tumors of human breast cancer patients. To investigate its role in the pathobiology of breast cancer, seprase was specifically suppressed in MDA-MB-435 and MDA-MB-436 cells by expression of antisense seprase cDNA. Suppression of seprase in the stable antisense transfectants was confirmed by immunoblotting, zymography, and fluorescence-activated cell sorting of cells labeled with antibody to seprase. Control-transfectants continued to express high levels of seprase. Seprase-suppressed MDA-MB-435 cells growing on type I collagen gels reveal a markedly different morphology than the parental or control-transfected cells that express high levels of seprase. The seprase-suppressed cells grow in islands and aggregates of tightly attached cells while those cells with high seprase grow as groups of separate individual cells. Seprase suppressed MDA-MB-435 and MDA-MB-436 breast cancer cells also exhibit altered growth properties. Seprase-suppressed cells and those with high seprase proliferate in serum-containing media. However, seprase-suppressed cells do not proliferate over 48 h of serum starvation, while cells with high seprase continue to proliferate. Cell cycle analysis of serum-starved cells reveal that seprase suppressed cells accumulate in G_2. These findings suggest that

seprase promotes the growth of breast cancer cells. Supported by DoD, Department of the Army, grant DAMD17-96-1-6097.

Bibliography of all publications and abstracts

3 Papers:

Kelly, T., Y. Yan, R.L. Osborne, A.B. Athota, T.L. Rozypal, J.C. Colclasure, and W.S. Chu (1998). Proteolysis of extracellular matrix by invadopodia facilitates human breast cancer cell invasion and is mediated by matrix metalloproteinases. Clin. Exp. Metastasis. *16*:501-512.

Kelly, T., S. Kechelava, T.L. Rozypal, K.W. West, and S. Korourian (1998). Seprase, a membrane-bound protease, is overexpressed by invasive ductal carcinoma cells of human breast cancers. Modern Pathol. *11*:855-863.

Kelly, T. (1999). Evaluation of seprase activity. Clin. Exp. Metastasis. *17*:67-72.

1 Manuscript in preparation

Goodman, J.D., Rozypal, T.L., and T. Kelly (2001) Seprase promotes growth of human breast cancer cells. In preparation

3 Abstracts:

Kelly, T., R.L. Osborne, and Y. Yan. (1996). The invasion potential of human breast cell lines correlates with their proteolysis of extracellular matrix. "Proteases and Protease Inhibitors", an American Association for Cancer Research Special Conference, March 1-5, 1996, Panama City Beach, FL.

Kelly, T., Y. Yan, R.L. Osborne, A.B. Athota, T.L. Rozypal, and W.S. Chu. (1997). Human breast cancer cells must proteolytically degrade extracellular matrix to invade. DoD, U.S. Army Med. Res. and Mat. Com., Breast Cancer Research Program: "An Era of Hope", 10/31-11/4/1997, Washington, DC.

Rozypal, T.L., J.D. Goodman, and T. Kelly. (2000) Overexpression of seprase by human breast cancer cells facilitates their invasion of extracellular matrix. DoD, U.S. Army Med. Res. and Mat. Com., Breast Cancer Research Program: "An Era of Hope", 6/8-6/11/00, Atlanta, GA

Conclusions:

This past year, and the past four years of support have been successful and the goals of the project have been completed. Specifically, we have cloned full-length seprase cDNAs from the MDA-MB-436 human breast cancer cell line. DNA sequence analysis has confirmed the clones and enabled the production of seprase specific cDNA probes. Seprase specific probes directly demonstrated that seprase is over-expressed by at least some human breast cancer cell lines. The sequence analysis also reveals that seprase from human breast cancer is identical to that expressed by human melanoma cells and also to the previously cloned FAP-α. The fact that seprase is expressed by wild type MDA-MB-436 cells has been demonstrated by both immunoblot analysis with F19 monoclonal antibody to seprase/ FAP-α, by identification of seprase activity using gelatin zymography and by F19 surface labeling of MDA-MB-436 cells as detected by FACS. Investigation into the role of seprase in breast cancer invasion and metastasis has been completed both in vivo using the nude mouse model and in vitro. The results of these experiments were inconclusive but suggested that seprase does not play a major role in facilitating invasion and metastasis. However, comparisons of seprase suppressed breast cancer cells to their seprase expressing counterparts revealed unexpected roles for seprase in breast cancer. A role in promoting the growth of breast cancer cells is the best characterized of these new findings. Breast cancer cells that express seprase can grow in the absence of serum, but those that have seprase suppressed fail to grow without serum. The seprase-suppressed breast cancer cells grown without serum become growth arrested in G2 phase of the cell cycle. Future studies will be performed to determine the molecular mechanisms underlying the growth arrest of the seprase suppressed cells. A second new finding was the observed differences in cell morphology between seprase expressing and seprase suppressed cells grown on type I collagen gels. The aggregated and adherent appearance of the seprase suppressed cells suggested a reversion of these cells to a more normal phenotype. Finally, the seprase suppressed breast cancer cells more sensitive to doxorubicin. It will be interesting to examine the reason for the lack of growth observed for seprase suppressed cells treated with 3 μM doxorubicin. Specifically, it will be necessary to determine if doxorubicin causes the G2 arrest in proliferating cells observed for serum-starved cells. So far the work has resulted in three papers, one manuscript, and three abstracts. Thus, this research was productive and provided new information about seprase and its role in breast cancer.

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Appendix

Role of seprase in breast cancer invasion
Final report
March 30, 2001

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Includes:
7 text figures and figure legends.

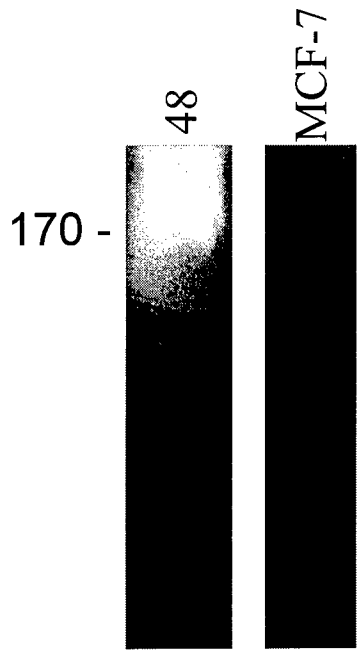


Figure 1. A) MCF-7 human breast cancer cells transfected with seprase cDNA express proteolytically active seprase. The 170 kDa gelatinase activity of seprase is readily detected in extracts of MCF-7 transfected with human breast cancer seprase cDNA from clone 48 (48). Seprase activity is not detected in extracts of non-transfected MCF-7 cells (MCF-7). Molecular weights are given x 10^{-3}.

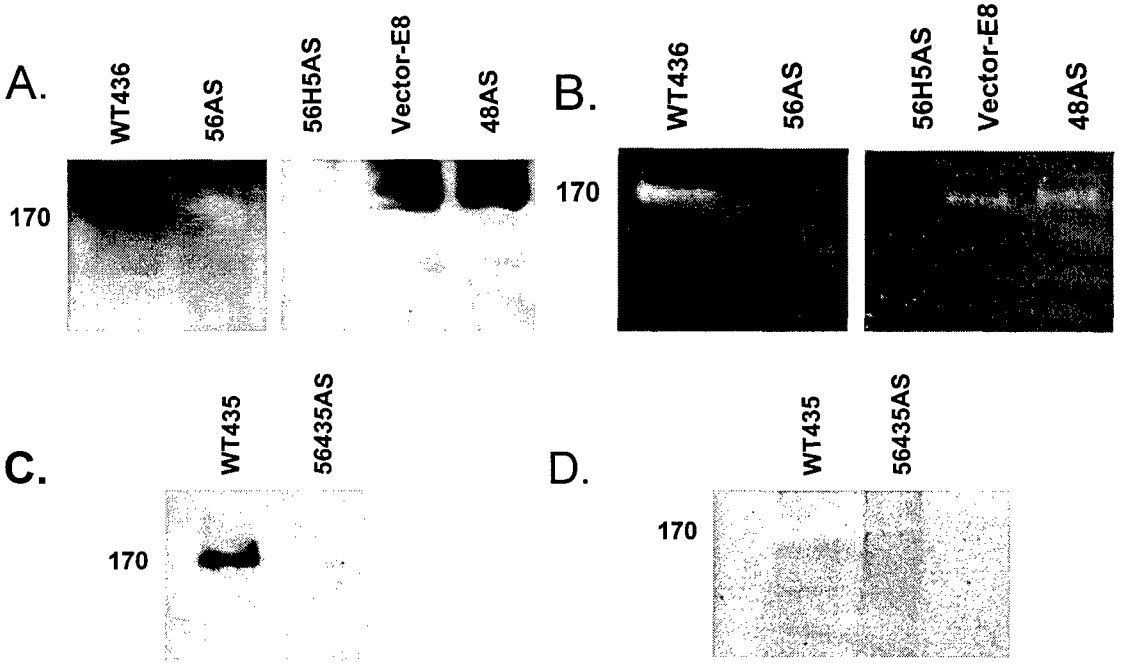


Figure 2. Suppression of seprase expression in MDA-MB-436 and MDA-MB-435 human breast cancer cells transfected with seprase antisense seprase cDNA.
A. Seprase (170 kDa) is detected with F19 mAb in extracts of parental MDA-MB-436 cells (WT436), control transfectants (Vector-E8), and antisense transfectants that failed to reduce seprase (48AS) of MDA-MB-436 cells. The 170 kDa seprase band is not detected by F19 in extracts of cells transfected with antisense clone 56 seprase cDNA (56AS) or a clonal transfectant of these cells (56H5AS).
B. Zymography reveals 170 kDa seprase activity in the extracts of parental MDA-MB-436 cells (WT436), control transfectants (Vector-E8), and antisense transfectants (48AS) of MDA-MB-436 cells. The 170 kDa seprase activity is not detected in extracts of cells transfected with antisense clone 56 seprase cDNA (56AS) or a clonal transfectant of these cells (56H5AS). Molecular weights are given x 10^{-3}.
C. High levels of seprase (170 kDa) are detected with F19 mAb in extracts of parental MDA-MB-435 cells (WT435). Reduced levels of the 170 kDa seprase band are detected by F19 in extracts of cells transfected with antisense clone 56 seprase (56435AS).
D. Zymography reveals 170 kDa seprase activity in the extracts of parental MDA-MB-435 cells (WT435). Reduction in the 170 kDa seprase activity is revealed in extracts of cells transfected with antisense clone 56 seprase cDNA (56435AS). Molecular weights are given x 10^{-3}.

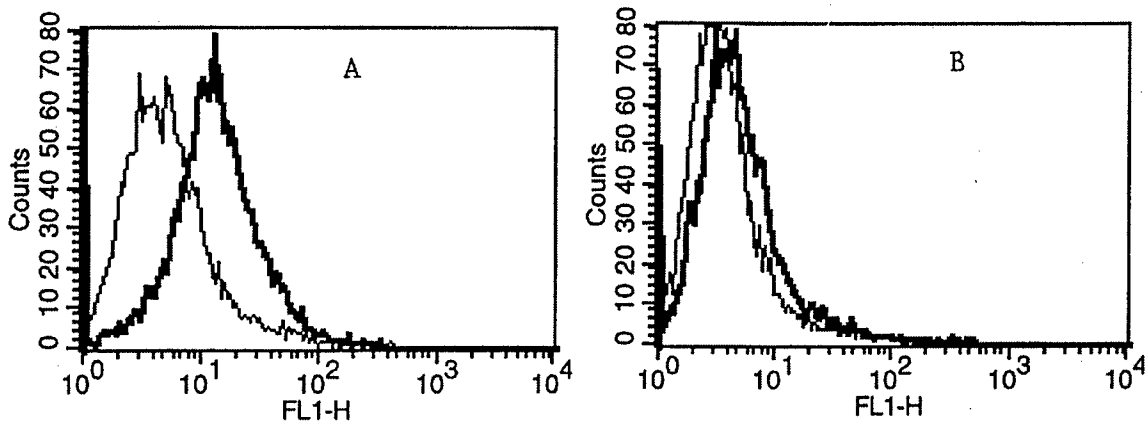


Figure 3. Antisense suppression of cell-surface seprase in MDA-MB-435 human breast cancer cells. A. FACS analysis of parental MDA-MB-435 human breast cancer cells stained with F19 or without F19 show high levels of seprase. B. MDA-MB-435 cells transfected with antisense seprase clone 56 have lower seprase expression.

Figure 4. Seprase suppressed cells form tight colonies and balls of cells on type I collagen gels. A. Seprase expressing MDA-MB-435 breast cancer cells grow on type I collagen gels as individual cells or in aggregates of cells where the membranes of individual cells are well demarcated. **B.** Seprase suppressed cells grow on type I collagen gels as tight aggregates with little separation of the individual cells. Balls of large numbers of cells are frequently seen (arrows).

Figure 4

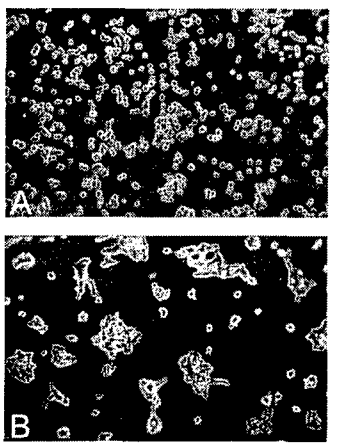
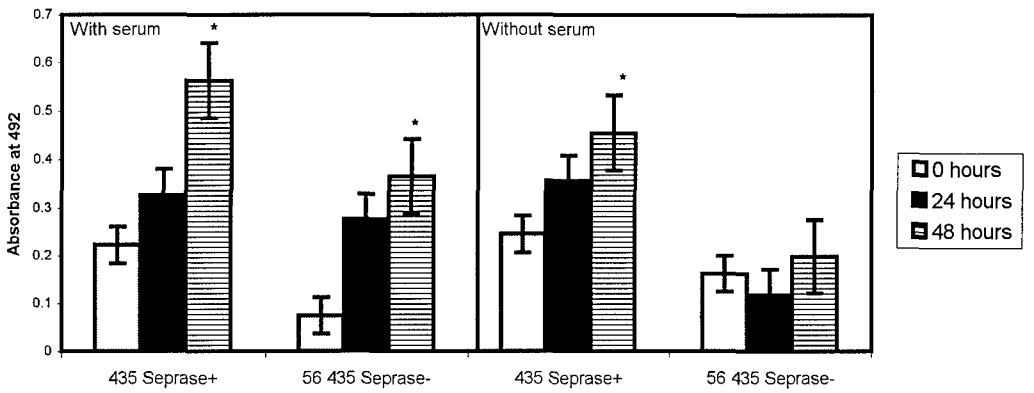


Figure 5. Seprase suppressed cells are sensitive to serum-starvation.
A) Cell proliferation assay reveals that both seprase expressing (435 BC) and seprase suppressed (56 435 BC) MDA-MB-435 cells proliferate in serum containing medium. Under serum-free growth conditions MDA-MB-435 expressing seprase proliferate (435 SF). Seprase-suppressed cells stop proliferating over 48 h in serum-free medium (56 435 SF). The cessation of growth of seprase suppressed cells is detected as no significant difference in cell number relative to time zero. Significance is noted by an asterisk and compares the 0 h time point to the 48 h time point (*); $p<.05$.

B) Cell proliferation assay reveals in the left panel that both seprase expressing (436 seprase high) and seprase suppressed (56H5 436 seprase low) MDA-MB-436 cells proliferate in serum containing medium over 72 h. Under serum-free growth conditions MDA-MB-436 expressing seprase proliferate slowly over the 72 h. time period (436) to achieve a greater number of cells that is significantly different from the number of cells determined at the beginning of the experiment (*); $p<.05$. Seprase-suppressed cells (56H5 436 seprase low) do not grow over the 72 h time period and the difference between number of cells at the beginning of the experiment is not different from that at the end.

Figure 5



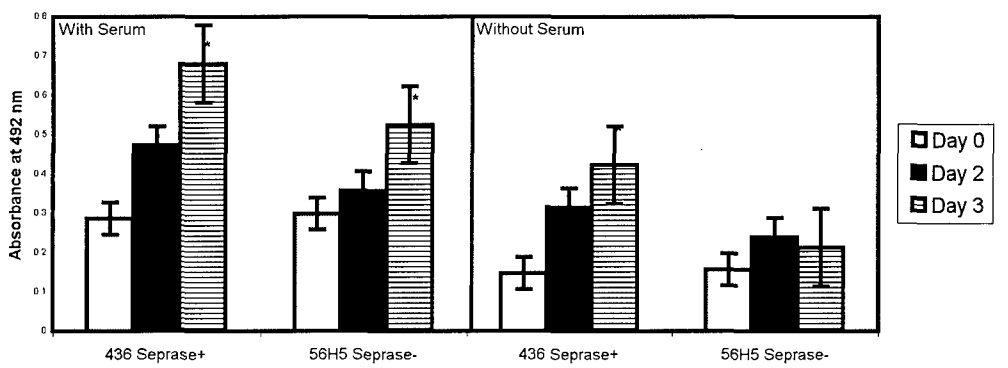
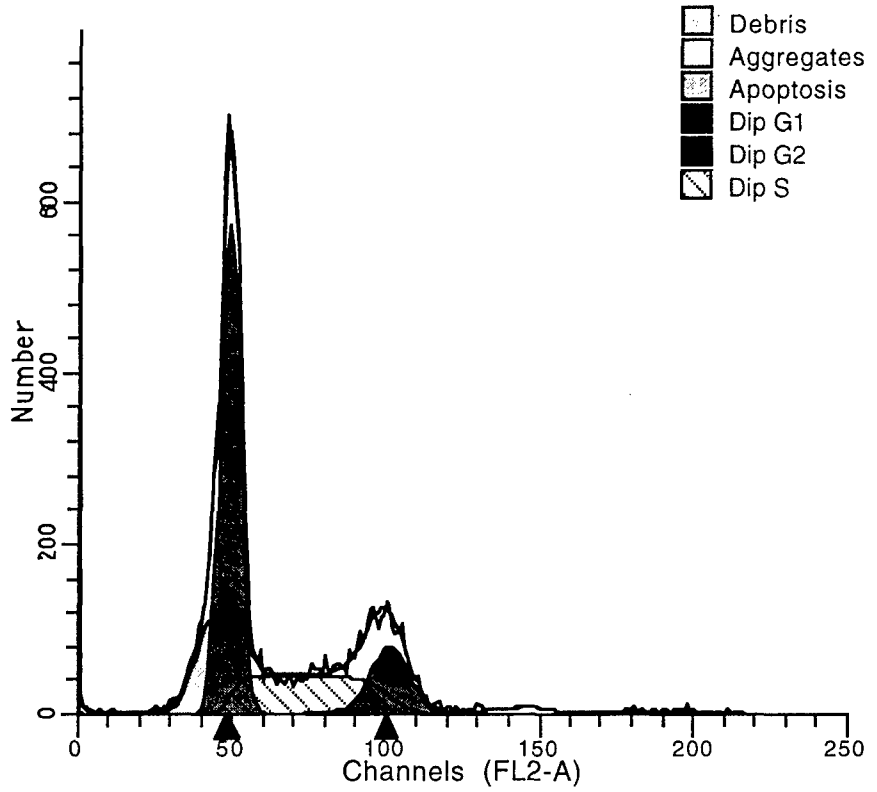


Figure 5

Figure 6. Serum starvation causes seprase-suppressed cells to growth arrest in G2. Effect of serum starvation on MDA-MB-436 cells expressing wild type seprase over 24 h (A) and 48 h (B) and on seprase suppressed transfectants 56436 over 24 h (C) and 48 h (D) and 56H5 over 24 h (E) and 48 h (F). Cells were subjected to DNA content analysis by propidium iodide staining and FACS by stabdard methods. In each histogram, the left arrow head at approximately 50, indicates 2N DNA content and G1. The next arrowhead to the right at approximately 100 indicates 4N DNA content and G2. Percentage of cells in different phases of the cell cycle or apoptosis are given on the top right of each histogram.

Figure 6A



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Date analyzed: 8-Mar-2001
Model: 1DA0A_DSF
Analysis type: Manual analysis

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Dip G1: 55.90 % at 49.07
Dip G2: 15.50 % at 101.05
Dip S: 28.60 % G2/G1: 2.06
%CV: 6.34

Total S-Phase: 28.60 %
Total B.A.D.: 7.29 %

Apoptosis: 14.95 % Mean: 43.59

Debris: 3.19 %
Aggregates: 8.42 %
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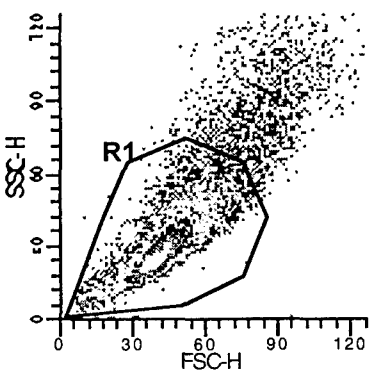
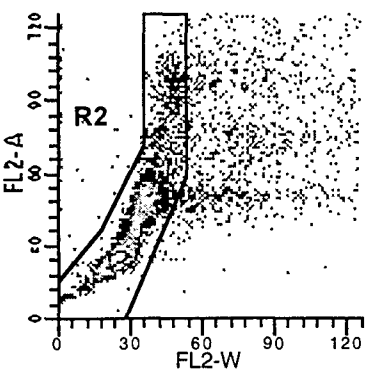
RCS: 1.975

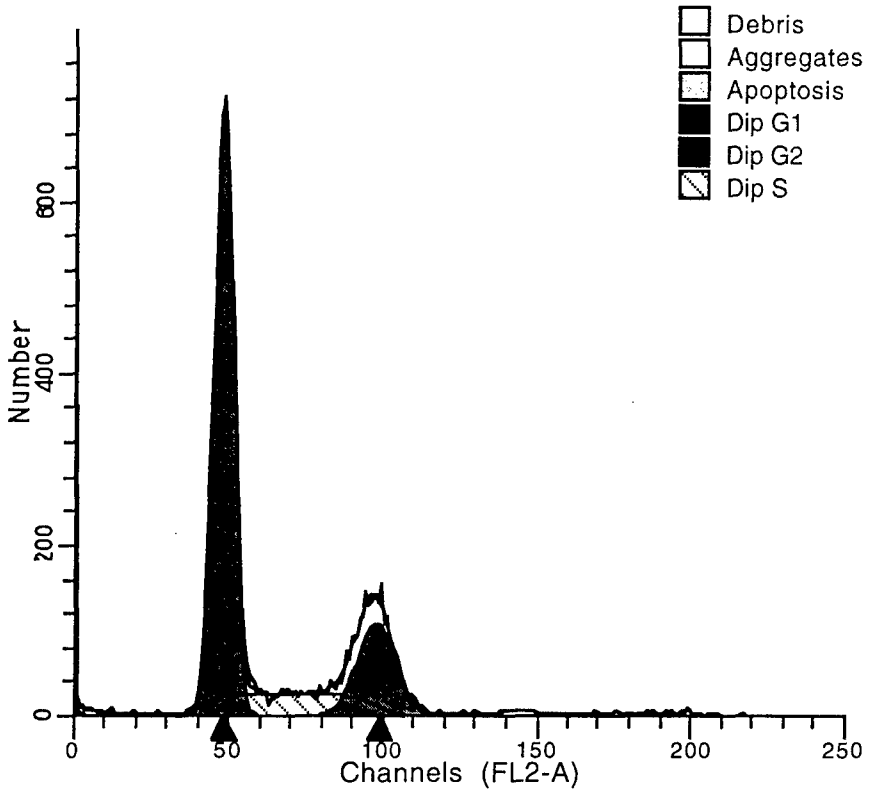




ModFitLT V3.0(PMac)

Figure 6A

Figure 6B



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Analysis type: Manual analysis

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Dip G2: 20.06 % at 97.36
Dip S: 15.64 % G2/G1: 2.03
%CV: 6.34

Total S-Phase: 15.64 %
Total B.A.D.: 5.58 %

Apoptosis: 0.05 % Mean: 27.51

Debris: 2.58 %
Aggregates: 7.24 %
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RCS: 1.854

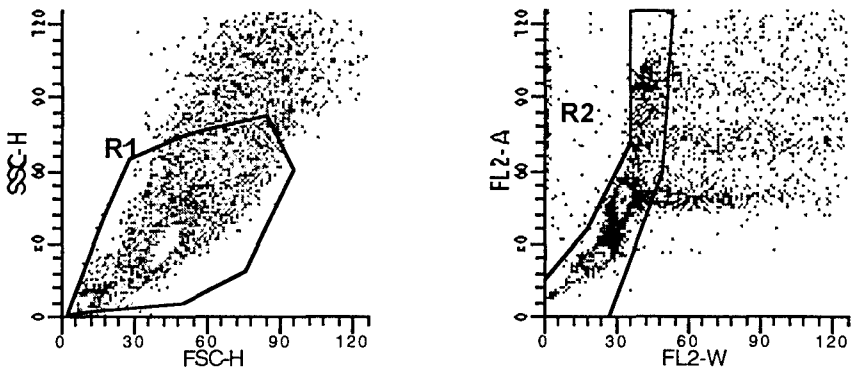

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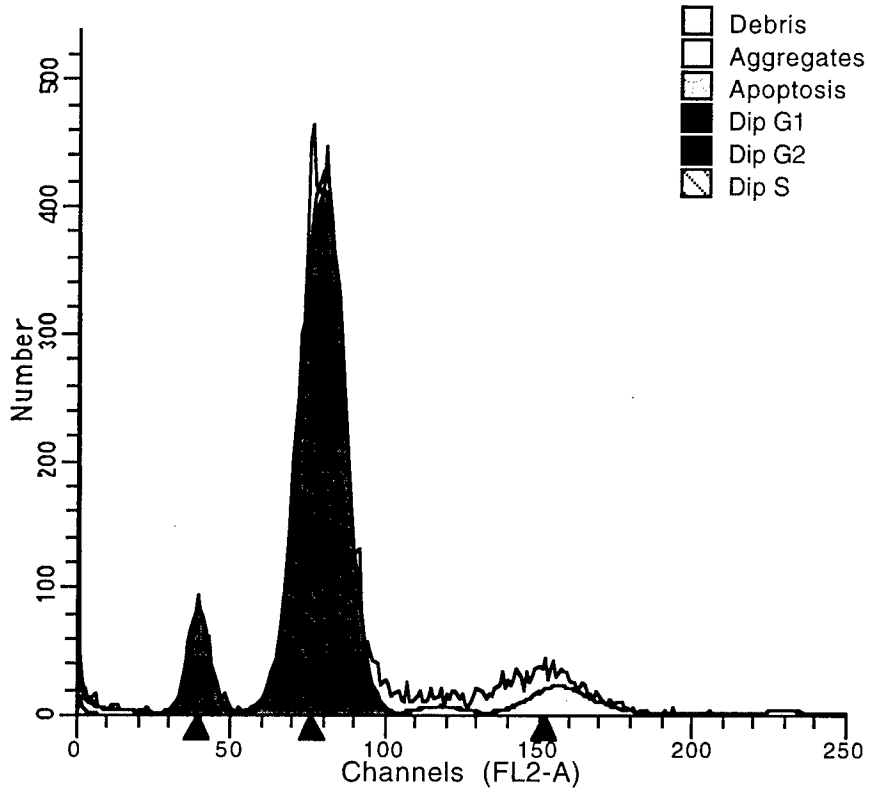
Figure 6B



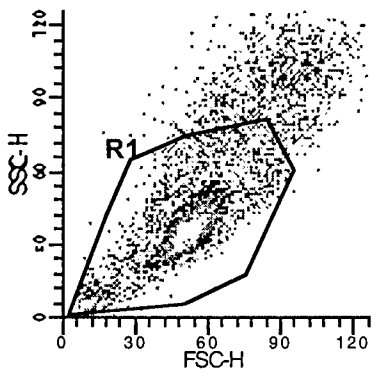
File analyzed: 030801.002
Date analyzed: 8-Mar-2001
Model: 1DA0A_DSF
Analysis type: Manual analysis

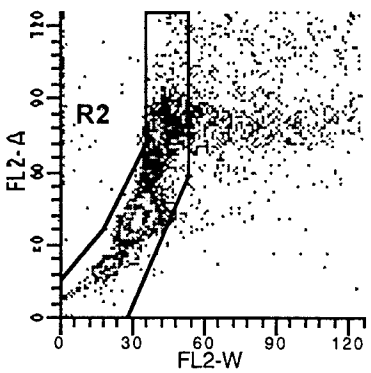
Diploid: 100.00 %
Dip G1: 8.42 % at 39.61
Dip G2: 91.33 % at 79.13
Dip S: 0.25 % G2/G1: 2.00
%CV: 8.78

Total S-Phase: 0.25 %
Total B.A.D.: 5.83 %

Apoptosis: 0.00 % Mean: 30.28

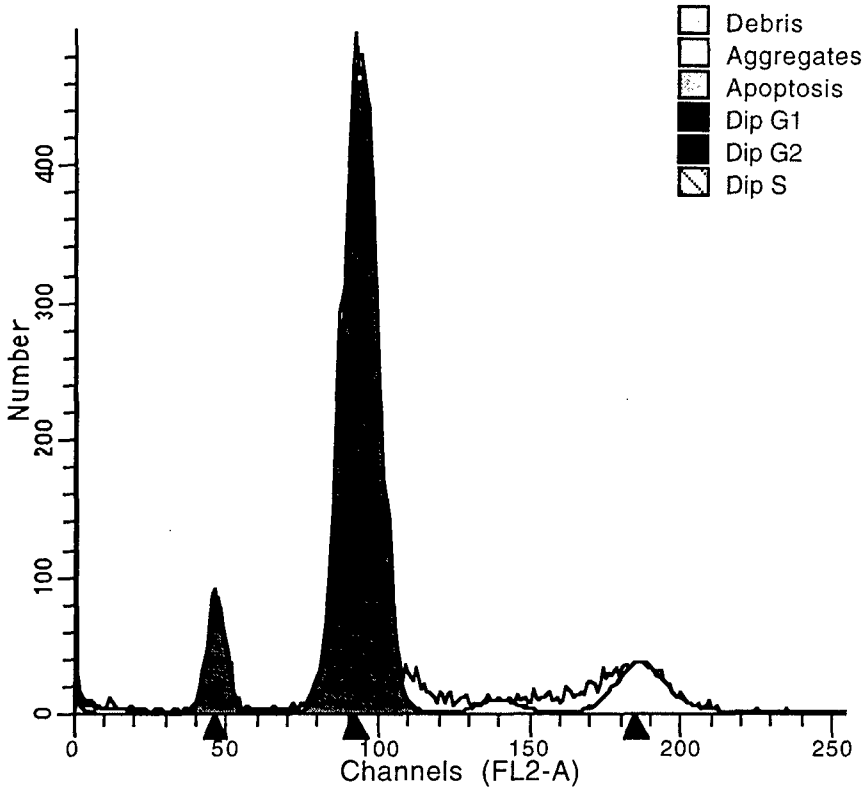
Debris: 3.90 %
Aggregates: 13.21 %
Modeled events: 9502
All cycle events: 7877
Cycle events per channel: 194
RCS: 5.489





ModFitLT V3.0(PMac)

Figure 6D



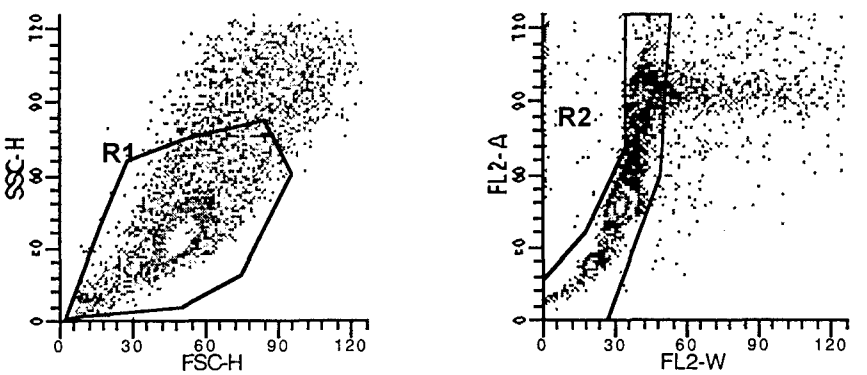
File analyzed: 030801.005
Date analyzed: 8-Mar-2001
Model: 1DA0A_DSF
Analysis type: Manual analysis

Diploid: 100.00 %
Dip G1: 7.86 % at 46.52
Dip G2: 91.56 % at 93.55
Dip S: 0.58 % G2/G1: 2.01
%CV: 6.12

Total S-Phase: 0.58 %
Total B.A.D.: 7.54 %

Apoptosis: 0.00 % Mean: 36.58

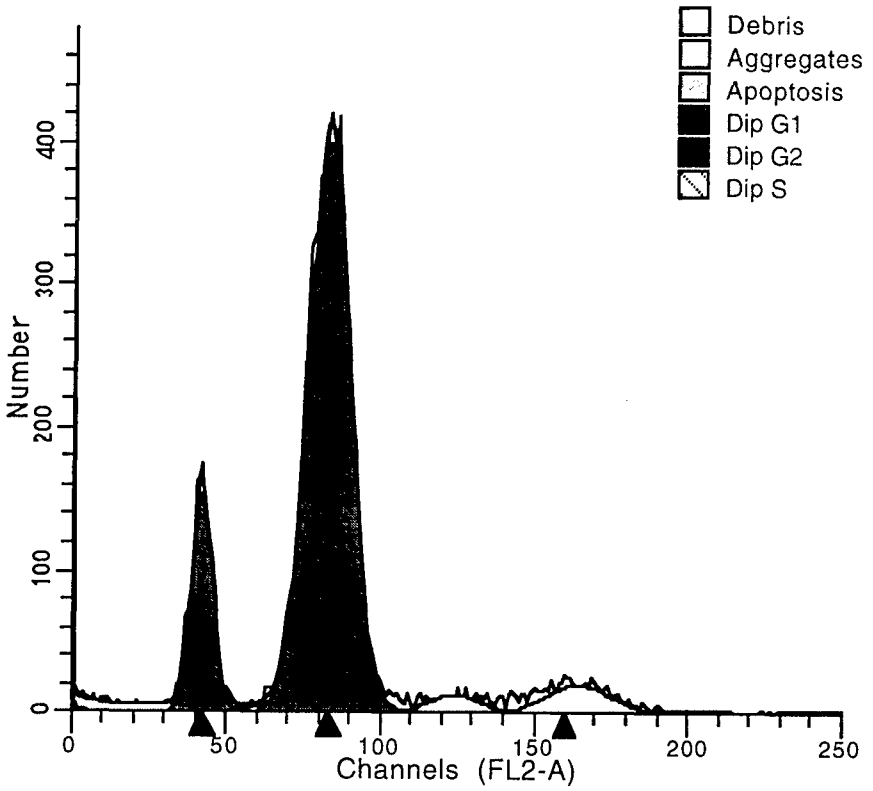
Debris: 4.34 %
Aggregates: 16.49 %
Modeled events: 9123
All cycle events: 7222
Cycle events per channel: 150
RCS: 3.641



ModFitLT V3.0(PMac)

Figure 6D

Figure 6E



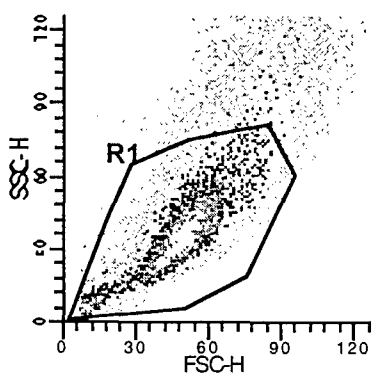
File analyzed: 030801.003
Date analyzed: 8-Mar-2001
Model: 1DA0A_DSF
Analysis type: Manual analysis

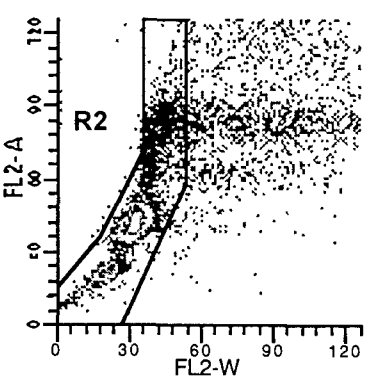
Diploid: 100.00 %
Dip G1: 16.16 % at 41.85
Dip G2: 83.56 % at 82.61
Dip S: 0.29 % G2/G1: 1.97
%CV: 7.96

Total S-Phase: 0.29 %
Total B.A.D.: 6.36 %

Apoptosis: 0.00 % Mean: 31.48

Debris: 4.76 %
Aggregates: 11.43 %
Modeled events: 9534
All cycle events: 7990
Cycle events per channel: 191
RCS: 2.163

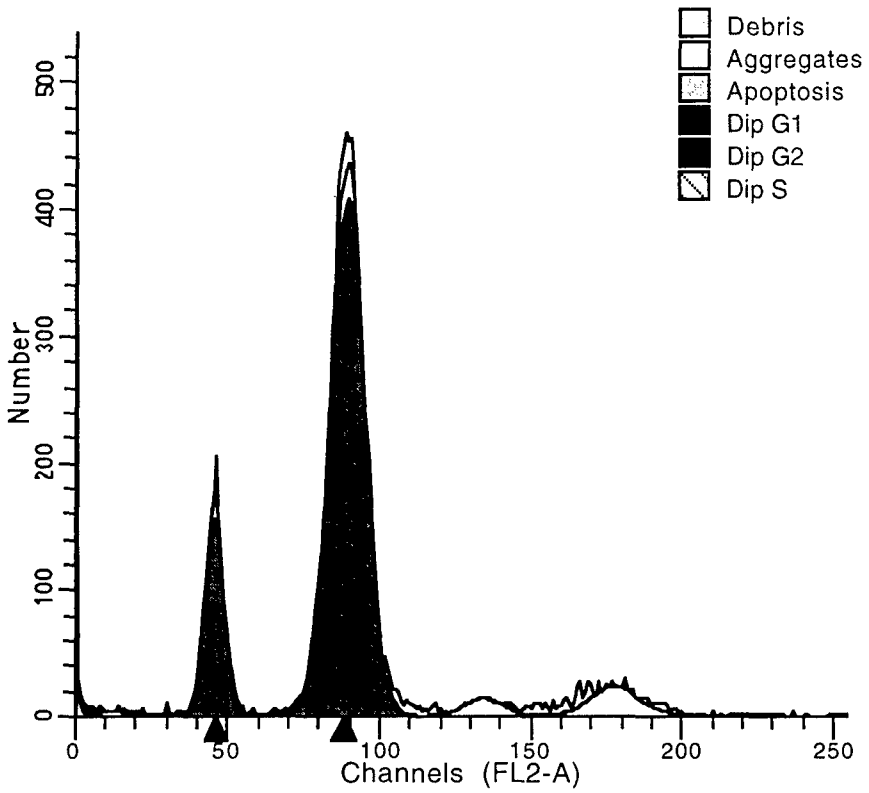




ModFitLT V3.0(PMac)

Figure 6E

Figure 6F



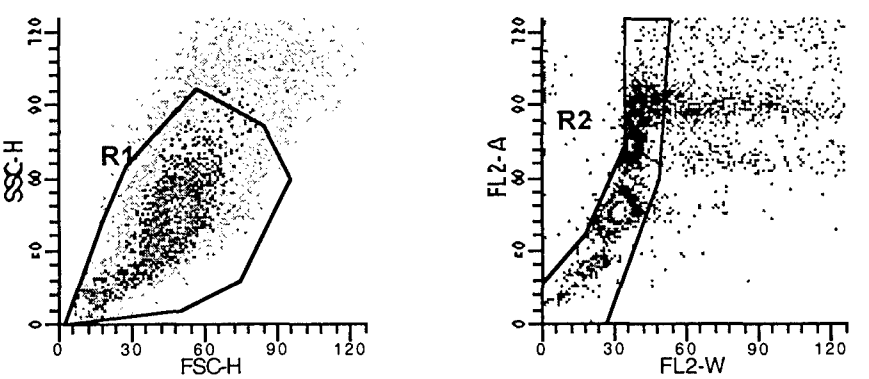
File analyzed: 030801.006
Date analyzed: 8-Mar-2001
Model: 1DA0A_DSF
Analysis type: Manual analysis

Diploid: 100.00 %
Dip G1: 16.34 % at 45.55
Dip G2: 83.42 % at 89.12
Dip S: 0.24 % G2/G1: 1.96
%CV: 6.13

Total S-Phase: 0.24 %
Total B.A.D.: 8.93 %

Apoptosis: 0.00 % Mean: 26.20

Debris: 3.91 %
Aggregates: 15.99 %
Modeled events: 8573
All cycle events: 6868
Cycle events per channel: 154
RCS: 2.432

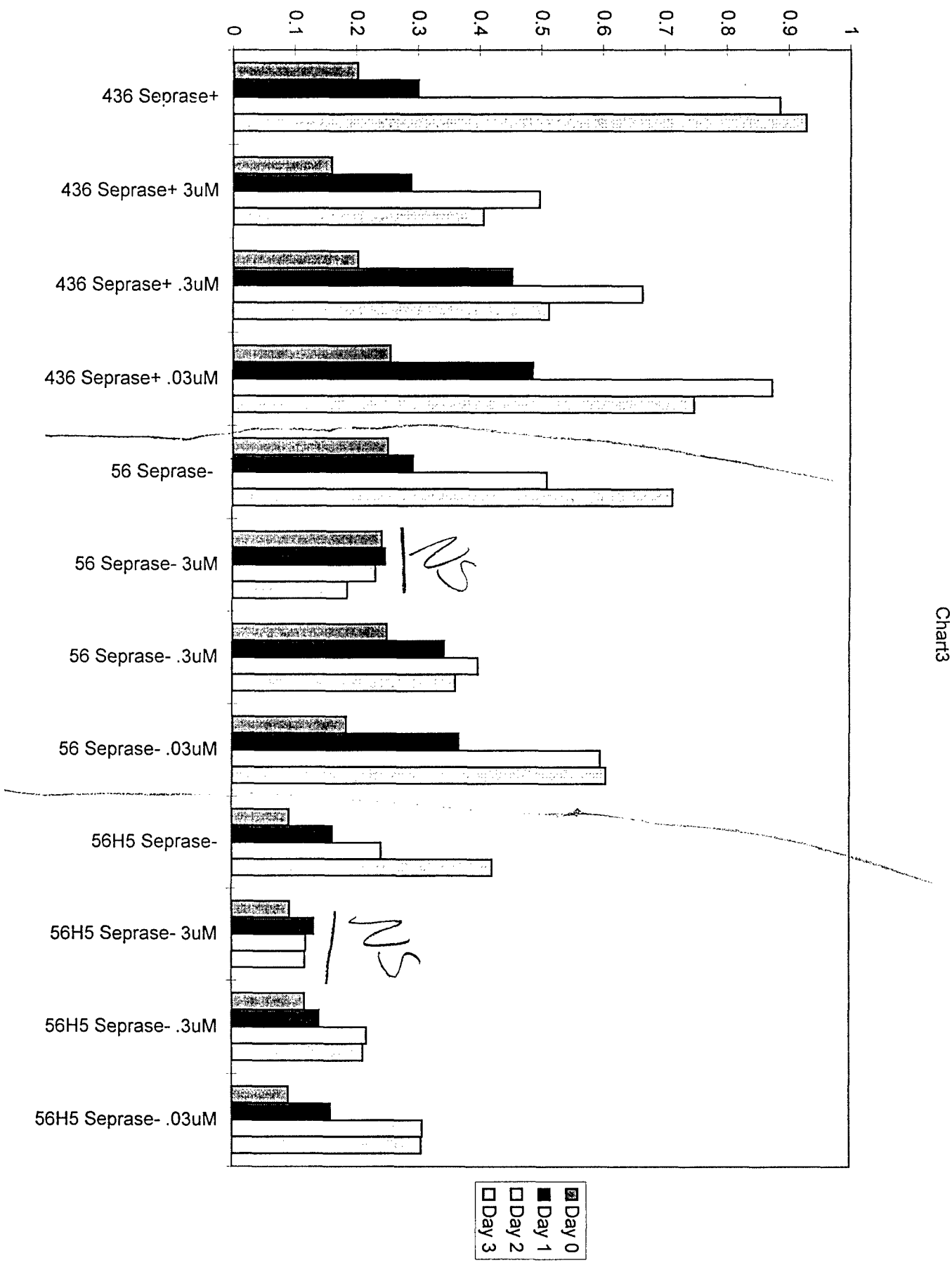


ModFitLT V3.0(PMac)

Figure 6F

Figure 7. Seprase suppressed cells are sensitive to doxorubicn Cell proliferation assay reveals that seprase expressing (436 seprase+) MDA-MB-436 cells proliferate in serum containing medium (436 Seprase) and serum containing medium to which 3, 0.3 or 0.03 μM doxorubicin has been added (436 seprase+ 3μM; 436 seprase+ .3 μM; and 436 seprase+ 0.03 μM). Seprase suppressed MDA-MB-436 also proliferate in serum containing medium (56 seprase-). The seprase suppressed 56 436 cells are growth inhibited by 3 μM doxorubicin (56 seprase- 3 μM, "NS" means no statistically significant difference in number of cells at time 0 or any of the later time points) but proliferate in presence of 0.3 μM and 0.03 μM doxorubicin (56 seprase- 0.3 μM; 56 seprase- 0.03 μM). The 56H5 seprase suppressed clonal transfectant MDA-MB-436 grows slowly in serum containing medium (56H5 Seprase-) but shows similar sensitivity to doxorubicin as the 56 436 cells. The 56H5 seprase suppressed cells do not proliferate in 3 μM doxorubicin (56H5 seprase-3 μM, "NS" means no statistically significant difference in number of cells at time 0 or any of the later time points) but are able to proliferate increasingly in lower concentrations of doxorubicin (56H5 seprase- 0.3 μM; 56H5 seprase- 0.03 μM). Except where noted by "NS", all cell viability at 0 through 72 hours were significantly different $p \leq 0.05$.

Figure 7


0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
436 Seprase+
436 Seprase+ 3uM
436 Seprase+ .3uM
436 Seprase+ .03uM
56 Seprase-
56 Seprase- 3uM
56 Seprase- .3uM
56 Seprase- .03uM
56H5 Seprase-
56H5 Seprase- 3uM
56H5 Seprase- .3uM
56H5 Seprase- .03uM
Day 0
Day 1
Day 2
Day 3
Chart3

Recd 10/29/2001

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND AND FORT DETRICK
810 SCHRIEDER STREET, SUITE 218
FORT DETRICK, MARYLAND 21702-5000

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Oct 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for the Accession Document Numbers listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Enclosure